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Concentration-Dependent Inhibition of Development of GGT Positive Foci in Rat Liver by the Environmental Contaminant Di(2-ethylhexyl) Phthalate

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The ability of di(2-ethylhexyl) phthalate (DEHP), a widely used plasticizer and environmental contaminant, to suppress development of putative preneoplastic lesions in rat liver was evaluated. γ-Glutamyl transpeptidase-positive (GGT+) foci were initiated in the livers of Sprague-Dawley male rats with a single dose of diethylnitrosamine (DEN) following partial hepatectomy. Promotion of foci was commenced by feeding a choline-deficient diet (CD). A group of control rats was fed a choline-supplemented diet (CS). The ability of DEHP to suppress the emergence of GGT+ foci was evaluated by feeding additional groups of rats the CD diet containing either 0.1%, 0.5%, 1.0% or 2.0% DEHP. The CD diet promoted the number of GGT+ foci above levels in control livers. Inclusion of the plasticizer to the levels of 0.5%, 1.0% and 2.0% in the CD diet effectively inhibited the appearance of the foci. However, DEHP was unable to inhibit the promoting effect of the CD diet at a concentration of 0.1%. DEHP's ability to block development of GGT+ foci correlated with its ability to increase liver weight and to induce carnitine acetyltransferase (EC 2.3.1.7), a marker of peroxisome proliferation.

Introduction

The diesters of *o*-phthalic acid are used to impart flexibility to plastics and may comprise up to 60% by weight of the finished product (*1*). DEHP, the most widely used plasticizer for polyvinyl chloride (PVC), had an estimated production volume of 280 million pounds in the United States for 1983 (*2*). This compound is not covalently bound to the PVC and can migrate out of the finished product with time and use. The large production volume coupled with the ability of the DEHP to migrate out of the plastic yield a large potential for human exposure. Although DEHP is relatively nontoxic, some concern about its safety has arisen because a recent study from the National Toxicology Program found that it increased the incidence of hepatocellular carcinoma and neoplastic nodules in rodents (*3*). Moreover, DEHP shares many biochemical properties with a class of compounds called peroxisome proliferators, two of which, nafenopin and Wy-14643, have been shown to promote the outgrowth of neoplastic foci in rat liver (*4,5*).

We recently examined the ability of DEHP to promote the outgrowth of GGT+ foci in rats previously administered a subcarcinogenic dose of diethylnitrosamine (*6*). The development of γ-glutamyl transpeptidase-positive (GGT+) foci has been closely linked to the subsequent development of both neoplastic nodules and hepatomas (*7–9*). Indeed, these foci are regarded by many as the precursor lesion for both neoplastic nodules and hepatomas (*8*). DEHP did not enhance the promotion of GGT+ lesions after either a 5 or 10 week feeding of the compound at a 2% level. Somewhat unexpectedly, DEHP suppressed the development of GGT+ foci by the well established promoting regimen, the choline- deficient diet (*6*). In a subsequent study we have also shown that DEHP at a 2% level blocked the development of GGT+ foci following initiation with DEN and promotion with phenobarbital (*10*). In the present study we have demonstrated that DEHP effectively suppressed development of GGT+ foci in the rat liver at levels as low as 0.5% and that this possible antipromoting activity correlated with its ability to induce the enzyme marker of peroxisome proliferation, carnitine acetyltransferase.

Materials and Methods

Male Sprague-Dawley rats weighing 150 to 200 g were housed individually in metal cages in a room with constant temperature and humidity and on a 12-hr light-dark cycle. The animals were fed a basal diet (Purina,

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Table 1. Number and size of foci and percent liver of GGT-positive hepatocytes.

Group[a]	Dietary treatment	No. of rats	Foci/cm^2	Area,[b] μ^2	% Liver as foci[c]
1	CS	3	4.2 ± 0.6[d] (2,4,6)[e]	12.5 ± 1.9 (2)	0.050 ± 0.005 (2,4)
2	CS+2% DEHP	3	0.8 ± 0.2	4.1 ± 0.6	0.003 ± 0.001
3	CD	3	12.9 ± 2.8 (1,2,4,5,6)	18.0 ± 3.2 (2)	0.24 ± 0.081 (2,4,5,6)
4	CD+2% DEHP	3	0.7 ± 0.2	6.2 ± 2.9	0.005 ± 0.004
5	CD+1% DEHP	5	1.6 ± 0.7	14.8 ± 8.8	0.025 ± 0.013
6	CD+0.5% DEHP	5	1.3 ± 0.7	18.7 ± 9.9	0.031 ± 0.017
7	CD+0.1% DEHP	5	6.6 ± 2.9	16.0 ± 3.2 (2)	0.119 ± 0.052

[a] All rats received 30 mg/kg DEN by IP injection at 18 hr after partial hepatectomy. Dietary treatment began 10 days after injection of DEN.
[b] Calculated from the average of the measured major and minor diameters assuming the shape of the focus to be an ellipse.
[c] The product of (foci/cm^2) × (avg. area) × 10^{-6}, where 10^{-6} is the reciprocal of the number of square microns per square centimeter times 100.
[d] Mean ± SE.
[e] $p < 0.05$ by Student t-test for two-tailed differences when compared with groups in parentheses ( ).

Ralston Purina Co., St. Louis, MO) and water *ad libitum* for at least 1 week before the start of experiments. Purified CS and CD diets were prepared according to the protocol of Shinozuka et al. (*11*).

For CD diets containing DEHP (Aldrich Chemical Co., Milwaukee, WI) the compound was added to levels of 0.1%, 0.5%, 1.0%, and 2.0% at the expense of sucrose. DEHP was added to a CS diet at a level of 2.0% to serve as a negative control. At 18 hr following subtotal hepatectomy, rats were administered a single intraperitoneal injection of DEN (Aldrich Chemical Co.) in saline at a dose of 30 mg/kg. Ten days later groups of animals (three to five rats/group) were placed on the experimental or control diets as indicated in Table 1. Animal weights and food consumption were monitored on two consecutive days at weekly intervals. DEHP intake for each group was calculated and averaged over the feeding period. Rats were sacrificed by carbon dioxide asphyxiation at 10 weeks following the commencement of the diets. Body weights and liver wet weights were determined. The livers were perfused with 50 mL ice-cold saline through the hepatic portal vein. Blocks of liver, which were quickly frozen on dry ice, had 8 μm cryostat sections cut and stained according to the procedure of Rutenburg et al. (*12*). GGT+ foci were counted and the major and minor diameter measured by an ocular micrometer. The area of each focus was estimated assuming it to be elliptical in shape (foci are rarely true circles). The area of the tissue section was measured using an Apple II+ computer with graphics tablet accessory. The size, number of foci per square centimeter, and the percent of tissue represented by the foci were calculated. Statistical comparisons were by Fisher F test and Student t test (*13*).

For use in the carnitine acetyltransferase (CAT) assay, the liver enzyme was solubilized according to the procedure of Moody and Reddy (*14*). All steps were carried out at 4°C. Briefly, a 5% homogenate was prepared in a buffer containing 0.25 M sucrose, 0.116 M Tris HCl, pH 8.0, and 0.0025 M EDTA. The homogenate was sonified for 2 min and allowed to stand overnight. The homogenate was then centrifuged at 76,300*g* for 1 hr. The resulting supernatant fraction was assayed for CAT activity by the method of Fritz and Schultz (*15*). The release of acetyl coenzyme A-SH in the presence of 5,5'-dithiobis (2-nitrobenzoate) was followed spectrophotometrically at 412 nm at 20°C, either in the presence or absence of carnitine.

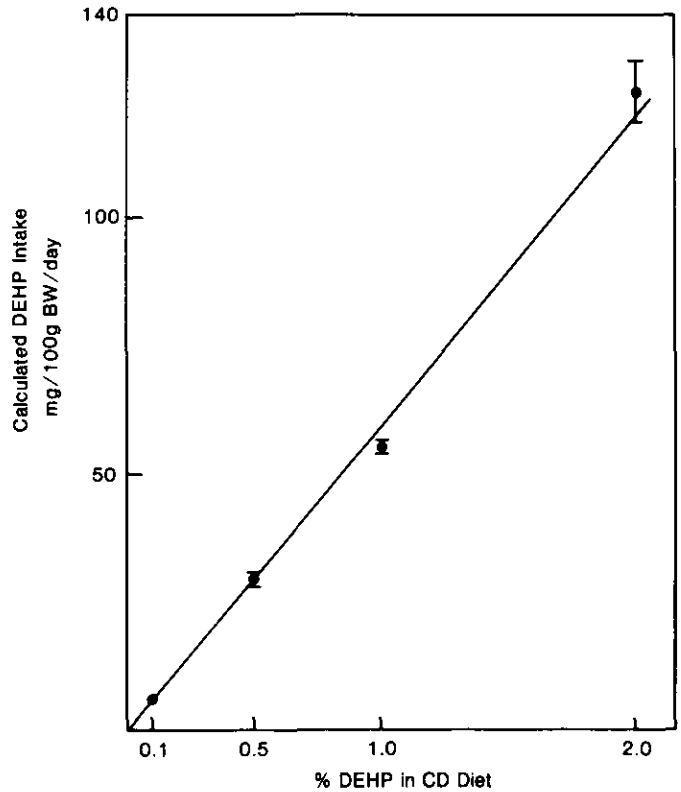

FIGURE 1. Dietary DEHP intake. DEHP intake (mg/100 g body weight) was calculated by measuring the consumption of CD diets containing 2.0%, 1.0%, 0.5%, and 0.1% of the plasticizer over a 24-hr period at weekly intervals.

Results

Promotion of foci was commenced by feeding the CD diet which effectively increased the number of GGT+ foci above levels in control rats fed only the CS diet (12.93 ± 2.76 vs. 4.16 ± 0.60 foci/cm, Table 1). To evaluate the antipromoting activity of DEHP, groups of rats received the CD diet containing 0.1%, 0.5%, 1.0% and 2.0% DEHP. Figure 1 shows the amount of DEHP ingested by each group based on recorded food consumptions. The amount of the plasticizer ingested increased linearly with the dietary concentration from 6 mg/100 g body weight (BW)/day for animals on the 0.1% diet to 119 mg/100 g BW/day for the 2.0% treated animals. This linearity reflected a constant food uptake between the groups of animals fed the CD diet containing the varying amounts of the plasticizer.

The number of foci developing in the livers of the rats on the various regimens is shown in Table 1. As can be seen, the CD + 2.0% DEHP regimen decreased the number of GGT+ foci from 12.93 ± 2.76/cm^2 to 0.83 ± 0.17 foci/cm^2 as would be expected from our earlier study (*6*). In addition, however, was the finding that plasticizer concentrations of 1.0% and 0.5% were equally effective in suppressing the development of foci (Table 1). The CD + 0.1% DEHP diet did decrease the mean value of the number of foci, but not to a statistically significant degree. In addition to decreasing the number of foci below that observed in rats on the CD diet alone, the CD diets containing 0.5%, 1.0% and 2.0% DEHP reduced the number of foci below that seen for the control rats on the CS diet (Table 1). We had observed a similar finding in which rats on the CS diet which had shown significantly more foci than those fed the CS + 2.0% DEHP diet (*6*). Likewise, in the present study the CS + 2.0% DEHP diet reduced the number of foci below that arising from endogenous promoting factors (41.6 ± 0.60 vs. 0.83 ± 0.1).

An additional important observation was that rats fed CD diets containing 1.0% and 0.5% DEHP had mean body weight gains exceeding that of the CD group (Fig. 2). In our previous studies, rats receiving the 2.0% DEHP diet had sometimes demonstrated a lower mean body weight gain than rats on the CD diet alone although the magnitude of the difference was not statistically significant. Nevertheless, since it had been reported that decreased body weights can be associated with diminished incidence of tumors (*16*), the lower weight gain in the DEHP-treated animals raised the remote possibility that the lower number of foci in DEHP treated rats might represent some type of nonspecific "starvation" effect. However, the finding that 0.5% and 1.0% concentrations of DEHP both inhibited foci formation and did not decrease total body weight gain has eliminated that possibility.

In order to independently demonstrate that DEHP was, in fact, exerting its usual biochemical effect in the

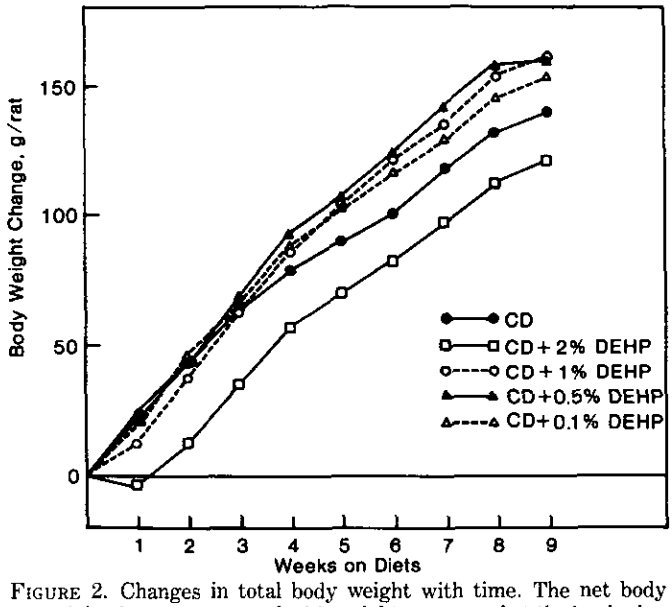

FIGURE 2. Changes in total body weight with time. The net body weight changes compared with weights measured at the beginning of the diets are shown.

Table 2. Carnitine acetyltransferase activity and liver weight changes.

Group[a]	Dietary treatment	No. of rats	CAT activity[b]	Liver weight, g/100 g body weight[c]
1	CS	3	0.00 ± 0.00[d]	3.56 ± 0.24
2	CS+2% DEHP	3	4.26 ± 0.86 (1,3,4,5,6,7)[d]	6.11 ± 0.35 (1,3,6,7)
3	CD	3	0.05 ± 0.03	3.56 ± 0.29
4	CD+2% DEHP	3	1.93 ± 0.19 (1,3,7)	5.61 ± 0.31 (1,3,6,7)
5	CD+1% DEHP	5	1.55 ± 0.33 (1,3,7)	4.95 ± 0.30 (1,3,7)
6	CD+0.5% DEHP	5	1.45 ± 0.11 (1,3,7)	4.59 ± 0.08 (1,3,7)
7	CD+0.1% DEHP	5	0.14 ± 0.07 (1)	4.12 ± 0.06

[a] All rats received 30 mg/Kg DEN by IP injection at 18 hr after partial hepatectomy. Dietary treatment began 10 days after injection of DEN.
[b] Carnitine acetyltransferase activity is expressed as μmole coenzyme ASH released/mg protein/min.
[c] Mean ± SE.
[d] $p < 0.05$ by Student t-test for two-tailed differences when compared with groups in parentheses ( ).

livers of animals fed either the CS or CD diets, we measured the induction of carnitine acetyltransferase (CAT). The activity of this enzyme parallels peroxisome proliferation (*14*), a process which is thought to be in part responsible for the alleged tumor inducing activity of DEHP (*17,18*). Little or no CAT activity was measured in the livers of rats fed the CS or CD diet alone (Table 2). Moreover, the CD + 0.1% DEHP diet which failed to inhibit the outgrowth of preneoplastic foci (Table 1) also failed to induce CAT activity above that of the CD diet. The CD diets containing 0.5%, 1.0%, and 2.0% DEHP and the CS + 2.0% DEHP diet all increased CAT activity above control levels. This effect of DEHP on liver CAT activity was also paralleled by its well known effect on liver weight. DEHP increased liver weight (g/100 g body weight) when present in the diet at 2.0%, 1.0%, and 0.5% (Table 2) but was ineffective at 0.1%. Thus, the ability of DEHP to inhibit the outgrowth of GGT+ foci was directly correlated with induction of CAT activity and increased liver weight, two well documented effects of the plasticizer.

Discussion

DEHP, while not genotoxic (*18,19*), was shown in one recent study to increase the incidence of liver tumors when fed at a dose of 1.2% in the diet of Fisher 344 rats (*3*) and in another study to increase the number of preneoplastic basophilic foci in the livers of mice given a subcarcinogenic dose of DEN (*20*). However, in earlier studies of this compound, it did not show a tumorigenic effect when incorporated into the diets of rats (*21,22*). In our studies (*6,10*), DEHP has consistently suppressed the development of putative preneoplastic GGT+ foci when administered in the diets of rats given a subcarcinogenic dose of DEN. The results in this report have shown that this effect was dose related and that it correlated directly with other known biochemical effects of DEHP. DEHP fed at levels of 0.5%, 1.0%, and 2.0% suppressed the emergence of preneoplastic foci while at a level of 0.1% it was not effective. Correspondingly, DEHP caused a significant increase in the activity of CAT, a marker of peroxisome proliferation (*14*) and liver weights at doses of the compound which inhibited the appearance of GGT+ foci but not at the level of 0.1% which was ineffective in suppressing promotion. This finding of a dose relationship adds further important evidence to our earlier studies suggesting that this compound may have antitumor promoting activity in the rat liver carcinogenesis model.

We have been interested in possible mechanisms which might explain DEHP's inhibition of GGT+ foci development. Foci presumably arise from carcinogen- initiated hepatocytes which have acquired the ability for "semi-autonomous" growth (*7,8*). Suppression of this growth may be due to DEHP's effects on membrane-bound enzymes. DEHP is lipophilic and can suppress the activity of HMG CoA reductase (3-hydroxy-3- methylglutaryl CoA reductase, EC 1.1.1.34) which is the enzyme responsible for the regulation of *de novo* cholesterogenesis (*23*). This action could provide one mechanism by which DEHP might suppress cell growth since intermediates from the synthesis of cholesterol appear to be required for cell division (*24,25*). We are currently studying another group of membrane-bound enzymes which appear to be affected by DEHP. These are the liver cell plasma membrane protein kinases (*26*). Our results indicate that chronic feeding of DEHP results in inhibition of the ability of epidermal growth factor to stimulate the autophosphorylation of its 175,000 plasma membrane receptor protein (*26*).

The reason for the apparent discrepancy between our results demonstrating possible antipromoting activity by DEHP and the results of others demonstrating tumorigenic or promoting activity for this compound and several other peroxisome proliferators is unclear at this juncture. Recently, the peroxisome proliferators, BR-931 and nafenopin, were shown to suppress the development of GGT+ foci in rats initiated with DEN (*27,28*). While differences in methodological detail were present between the studies showing promotion and those showing possible antipromotion, no obvious difference was noted which could explain the apparent discrepancy in results. One possible explanation could be that DEHP suppresses the development of those foci which would regress anyway, since most foci presumably do not develop into neoplastic nodules or hepatomas (*29*). Alternatively, if the development of neoplastic nodules and hepatomas proceeds through pathways other than the formation of GGT+ foci as has been suggested by some (*30*), then DEHP's ability to suppress development of GGT+ foci would not necessarily indicate a true antipromoting activity. We are currently extending our studies with DEHP to further clarify this question.

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